National Standard of the People's Republic of China

GB 4789.30- 2010

National food safety standard
Food microbiological examination: *Listeria monocytogenes*

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Foreword

This standard replaced GB/T4789.30 - 2003 *Microbiological Examination of Food Hygiene - Examination of Listeria Moncytogenes*.

Compared with GB/T 4789.30-2008, the following main changes have been made to this standard:

- The Chinese and English names are modified;
- The second method: Screening Method with Automatic Enzyme-linked Fluorescence Immuno-analyzer is deleted;
- The third method: Screening Method with Automatic Pathogenic Bacteria Detection System is deleted.

Appendix A of this standard is a normative appendix.

The original editions replaced by this present National Standard include:

- GB/T4789.30 – 1994, GB/T 4789.30 – 2003, and GB/T 4789.30-2008

National food safety standard
Food microbiological examination: *Listeria monocytogenes*

1 Scope

This standard specifies the testing methods of Listeria monocytogenes in foods.

This standard applies to testing of Listeria monocytogenes in foods.

2 Apparatus and materials

Except for routine sterilization and culture equipments in microorganism laboratories, other equipments and materials are as follows:

2.1 Refrigerator: 2℃ - 5℃.

2.2 Thermostatic incubator: 30℃±1℃, 36℃±1℃.

2.3 Homogenizer.

2.4 Microscope: 10× - 100×.

2.5 Electronic balance: with a reciprocal sensibility of 0.1g.

2.6 Conical flask: 100mL, 500mL.

2.7 Sterile pipette: 1mL (with 0.01ml graduation), 10ml (with 0.01ml graduation).

2.8 Sterile plate: with a diameter of 90mm.

2.9 Sterile test tube: 16mm×160mm.

2.10 Centrifuge tube: 30mm×100mm.

2.11 Sterile syringe: 1mL.

2.12 Staphylococcus aureus (ATCC25923).

2.13 Rhodococcus equi.

2.14 Mus musculus albus: 16g - 18g.

2.15 Automatic microorganism identification system.

3 Culture medium and reagents

3.1 Tryptone soya broth containing 0.6% yeast extract (TSB - YE): see section A.1 in Appendix A.

3.2 Trypticase soy agar containing 0.6% yeast extract (TSA - YE): see section A.2 in Appendix A.

3.3 Listeria enrichment broth LB (LB_1, LB_2): see section A.3 in Appendix A.

3.4 1% acriflavine HCl solution: see section A.3.2.1 in Appendix A.

3.5 1% nalidixate sodium (naladixic acid) solution: see section A.3.2.1 in Appendix A.

3.6 PALCAM agar: see section A.4 in Appendix A.

3.7 Gram staining solution: see section A.5 in Appendix A.

3.8 SIM motility culture medium: see section A.6 in Appendix A.

3.9 Buffer glucose peptone water [used for methyl red (MR) and V – P test]: see section A.7 in Appendix A.

3.10 5% - 8% goat blood agar: see section A.8 in Appendix A.

3.11 Sugar fermentation tube: see section A.9 in Appendix A.

3.12 Catalase test: see section A.10 in Appendix A.

3.13 Listeria chromagenic medium

3.14 Biochemical identification kit.

4 Test program

For the procedures for examination of Listeria monocytogenes, please see figure 1.

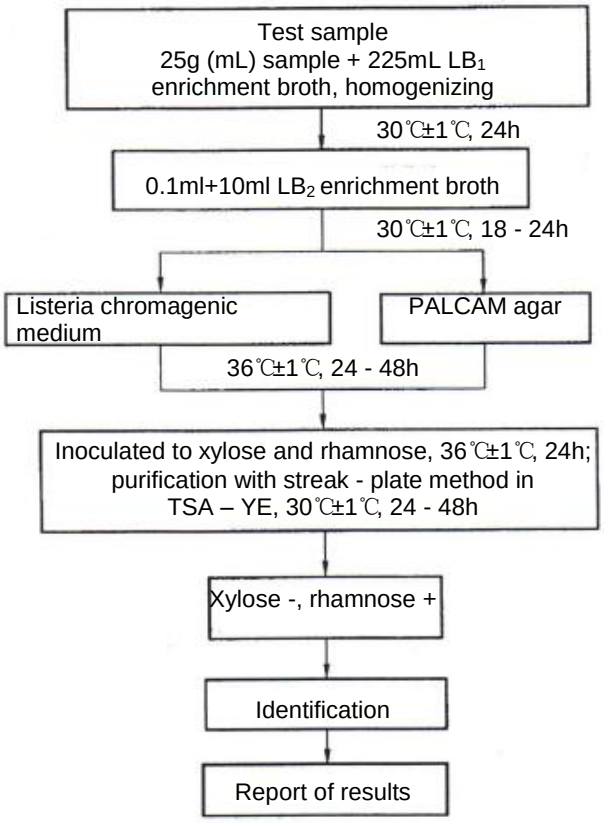

Figure 1 The procedures for examination of Listeria monocytogenes

5 Procedures

5.1 Enrichment

Transfer 25g (mL) sample with aseptic techniques to a homogenizing bag containing 225ml LB_1 enrichment broth, continuously homogenize it on a slapping homogenizer for 1 min - 2min; or place it to a homogenizing beaker containing 225ml LB_1 enrichment broth, and then homogenize it at 8000r/min -

10000r/min for 1min - 2min. Incubate it at 30℃±1℃ for 24h, transfer 0.1ml and inoculate it to 10ml LB_2 enrichment broth, and then incubate it at 30℃±1℃ for 18h - 24h.

5.2 Isolation

Take some LB_2 secondary enrichment broth and inoculate it to a PALCAM agar plate and Listeria chromagenic medium with streak method, incubate it at 36℃±1℃ for 24h - 48h, and then observe the colonies growing on each plate. The typical colonies on PALCAM agar plate are small round mignonette colonies surrounded by brownish-black hydrolysis halo, some colonies have black concave; and characteristic of the typical colonies on Listeria chromagenic medium are judged according to the instruction of product.

5.3 Preliminary screening

Pick more than 5 typical or suspected colonies from the selective agar plate and inoculate respectively in xylose and rhamnose fermentation tube, and then incubate it at 30℃±1℃ for 24h - 48h. Select pure colonies which are xylose-negative and rhamnose-positive and continue to carry out identification.

5.4 Identification

5.4.1 Microscopic examination of staining: Listeria is a kind of Gram positive rod bacteria, with the size of (0.4μm - 0.5μm) × (0.5μm - 2.0μm); prepare bacterial suspension with physiological saline, observe them under an immersion objective or a phase contrast microscope, this kind of bacteria will slightly revolve or roll.

5.4.2 Motility test: Listeria has motility; it grows with the shape of umbrella or crescent.

5.4.3 Biochemical identification: Pick up single pure suspected colony, and carry out catalase test on it; for the colonies which have positive reaction of catalase, continue to carry out sugar fermentation test and MR-VP test. For the main biochemical characteristics of Listeria monocytogenes, please see table 1.

5.4.4 Haemolysis test: Divide the bottom of goat blood agar plate to 20 – 25 grids, pick single pure suspected colony and then inoculate it to a blood plate with pin-prick inoculation method, each grid with one colony; inoculate positive control bacteria (Listeria monocytogenes and L.ivanovii) and negative control bacteria (L.innocua) with pin-prick inoculation; when pricking, the pin should be close to the bottom as far as possible, however, it shouldn't contact the bottom, and pay attention to avoid rupture of the agar; incubate it at 36℃±1℃ for 24h - 48h, and observe at a bright place. Listeria monocytogenes and L.seeligeri generate small transparent zone of hemolysis around the pin-prick inoculation spot, L.innocua doesn't generate zone of hemolysis, and L.ivanovii generate large transparent zone of hemolysis.

5.4.5 Cooperative hemolysis test (cAMP): Inoculate Staphylococcus aureus and Rhodococcus equi with parallel streaking on the goat blood agar plate; pick single pure suspected colony and inoculate it to the agar between the parallel lines with perpendicular streaking; the two ends of the vertical line shouldn't contact with the parallel lines, incubate it at 30℃±1℃ for 24h - 48h. Haemolysis by Listeria monocytogenes increases at the end with inoculation of Staphylococcus aureus and so does haemolysis of L.seeligeri, while haemolysis by L.ivanovii increases at the end close to the inoculation spot of

Rhodococcus equi.

5.5 Biochemical identification kit or Automatic microorganism identification system can be selected to identify 3 – 5 pure suspected colonies in section 5.3.

Table 1 The differences between biochemical characteristics of Listeria monocytogenes and those of other Listeria

Strain	Haemolysis reaction	Glucose	Maltose	MR - VP	Mannitol	Rhamnose	Xylose	Esculin
Listeria monocytogenes	+	+	+	+/+	-	+	-	+
L.grayi	-	+	+	+/+	+	-	-	+/+
L.seeligeri	+	+	+	+/+	-	-	+	+
L.welshimeri	-	+	+	+/+	-	V	+	+
L.ivanovii	+	+	+	+/+	-	-	+	+
L.innocua	-	+	+	+/+	-	V	-	+
Note: +: positive; -: negative; V: Response variable								

5.6 Virulence test in mice (Optional)

Inoculate the pure culture with the above-mentioned characristics to TSB - YE, incubate it at 30℃±1℃for 24h, centrifuge at 4000 r/min for 5, discard the supernatant, prepare it to bacterial suspension with a concentration of 10^{10}CFU/ml with sterile physiological saline, take the bacterial suspension and inject it peritoneally to 3 – 5 mice with 0.5ml/mouse, and then observe the death situation of mice. If pathogenic strain is inoculated, the mouse will die in 2d - 5d. During the test, a known strain can be used as a control. Listeria monocytogenes and L.ivanovii have pathogenicity for mice.

6 Report of results

According to the results of the above biochemical test and haemolysis test, report whether Listeria monocytogenes is detected or not detected in 25g (mL) sample.

Appendix A

(Normative)

Culture medium and reagents

A.1 Tryptone soya broth containing 0.6% yeast extract (TSB - YE)

A.1.1 Components

Tryptone	17.0g
Multivalent peptone	3.0g
Yeast extract paste	6.0g
Sodium chloride	5.0g
Dipotassium phosphate	2.5g
Glucose	2.5g
Distilled water	1000ml

pH 7.2～7.4

A.1.2 Preparation methods

Heat the above-mentioned components and stir to dissolve them, adjust pH, dispense the solution to tubes, autoclave at 121℃for 15 min, and then store it for later use.

A.2 Trypticase soy agar containing 0.6% yeast extract (TSA - YE)

A.2.1 Components

Tryptone	17.0g
Multivalent peptone	3.0g
Yeast extract paste	6.0g
Sodium chloride	5.0g
Dipotassium phosphate	2.5g
Glucose	2.5g
Agar	15.0g
Distilled water	1000ml

pH 7.2～7.4

A.2.2 Preparation methods

Heat the above-mentioned components and stir to dissolve them, adjust pH, dispense the solution to tubes, autoclave at 121℃for 15 min, and then store it for later use.

A.3 Listeria enrichment broth (LB_1, LB_2)

A.3.1 components

Tryptone	5.0g
Multivalent peptone	5.0g
Yeast extract paste	5.0g
Sodium chloride	20.0g

Potassium dihydrogen phosphate	1.4g
Dibasic sodium phosphate	12.0g
Esculin	1.0g
Distilled water	1000ml
pH 7.2～7.4	

A.3.2 Preparation methods

Heat the above-mentioned components and stir to dissolve them, adjust pH, dispense the solution to tubes, autoclave at 121℃for 15 min, and then store it for later use.

A.3.2.1 Add the following to 225ml Listeria Ⅰ solution (LB_1):

1% nalidixic acid (prepared with 0.05mol/L sodium hydroxide solution) 0.5mL

1% acridine yellow (prepared with sterile distilled water) 0.3ml

A.3.2.2 Add the following to 200ml Listeria Ⅱ solution (LB_2):

1% nalidixic acid	0.4 mL
1% acridine yellow (prepared with sterile distilled water)	0.5ml

A.4 PALCAM

A.4.1 Components

Yeast extract paste	8.0g
Glucose	0.5g
Esculin	0.8g
Ammonium ferric citrate	0.5g
Mannitol	10.0g
Phenol red	0.1g
Lithium chloride	15.0g
Pancreatic digest of casein	10.0g
Pancreatin digest of heart	3.0g
Corn starch	1.0g
Pepsin digest of meat	5.0g
Sodium chloride	5.0g
Agar	15.0g
Distilled water	1000ml
pH 7.2～7.4	

A.4.2 Preparation methods

Heat the above-mentioned components and stir to dissolve them, adjust pH, dispense the solution to tubes, autoclave at 121℃for 15 min, and then store it for later use.

A.4.2.1 PALCAM selective additive

Polymyxin B	5.0mg

Hydrochloric acidacridine yellow	2.5mg
Ceftazidime	10.0mg
Sterile distilled water	500ml

A.4.2.2 Preparation methods

Dissolve the PALCAM basic culture medium and then cool to 50℃, add to it 2ml PALCAM selective additive, mix it even, pour onto a sterile plate, and then store it for later use.

A.5 Gram staining solution

A.5.1 Crystal violet staining solution

A.5.1.1 Components

Crystal violet	1.0g
95% ethanol	20.0ml
1% ammonium oxalate water solution	80.0ml

A.5.1.2 Preparation methods

Dissolve crystal violet completely with ethanol and then mix it with ammonium oxalate solution.

A.5.2 Gram iodine solution

A.5.2.1 Components

Iodine	1.0g
Potassium iodide	2.0g
Distilled water	300.0ml

A.5.2.2 Preparation methods

Mix iodine and potassium iodide, add a little distilled water, shake well, and then add 300ml distilled water to the solution after it has completely dissolved.

A.5.3 Safranine re-staining solution

A.5.3.1 Components

Safranine	0.25g
95% ethanol	10.0ml
Distilled water	90.0ml

A.5.3.2 Preparation methods

Dissolve safranine with ethanol and then dilute it with distilled water.

A.5.4 Staining methods

A.5.4.1 Fix the smear of a single pure suspected colony on fire, add crystal violet staining solution drop by drop onto it, wait for 1min and then wash it with water.

A.5.4.2 Add Gram iodine solution drop by drop onto it, wait for 1min then wash it with water.

A.5.4.3 Add 95% ethanol drop by drop to destain, wait for about 15s - 30s until the staining solution is

washed away while it is not excessively destained, and then wash it with water.

A.5.4.4 Add re-staining solution drop by drop, restain for 1min, wash it with water and then observe under microscope when it is dry.

A.6 SIM motility culture medium

A.6.1 Components

Tryptone	20.0g
Multivalent peptone	6.0g
Ammonium ferric sulfate	0.2g
Sodium thiosulfate	0.2g
Agar	3.5g
Distilled water	1000ml

pH 7.2

A.6.2 Preparation methods

Heat the above-mentioned components and mix them even, adjust pH, dispense the solution to small test tube, autoclave at 121℃for 15 min, and then store it for later use.

A.6.3 Test methods

Pick single pure suspected colony and then inoculate it to SIM culture medium with pin-prick inoculation method, incubate it at 30℃for 24h - 48h, and then observe the results.

A.7 Buffer glucose peptone water (used for MR and V – P test)

A.7.1 Components

Poly peptone	7.0g
Glucose	5.0g
Dipotassium phosphate	5.0g
Distilled water	1000ml

pH 7.0

A.7.2 Preparation methods

Dissolve the above mentioned components and then adjust pH, dispense the solution to test tubes, 1ml per tube, autoclave at 121℃ for 15 min, and then store it for later use.

A.7.3 Methyl red (MR) test

A.7.3.1 Methyl red reagents

A.7.3.1.1 Components

Methyl red	10mg
95% ethanol	30ml
Distilled water	20ml

A.7.3.1.2 Preparation methods

Dissolve 10mg methyl red with 30ml 95% ethanol, and then add it to 20ml distilled water.

A.7.3.1.3 Test methods

Inoculate sufficient amount of agar culture to this culture medium, incubate it at 36℃±1℃ for 2d - 5d. Add one drop of methyl red reagent, and then observe the results immediately. If the color is bright red, then the result is positive; if the color is yellow, then the result is negative.

A.7.4 V – P test

A.7.4.1 6% α - naphthol - ethanol solution

Components and preparation methods: dissolve 6.0g α - naphthol with absolute alcohol and then dilute to 100ml.

A.7.4.2 40% potassium hydroxide solution

Components and preparation methods: dissolve 40g potassium hydroxide with distilled water then dilute to 100ml.

A.7.4.3 Test methods

Inoculate sufficient amount of agar culture to this culture medium, incubate it at 36℃±1℃ for 2d - 4d. Add 0.5ml 6% α - naphthol - ethanol solution and 0.2ml 40% potassium hydroxide solution, shake well, and then observe the results. If the reaction is positive, there will be red color appearing immediately or within several minutes; if the reaction is negative, incubate it at 36℃±1℃ for another 4h and then observe it.

A.8 Blood agar

A.8.1 Components

Peptone	1.0g
Beef extract	0.3g
Sodium chloride	0.5g
Agar	1.5g
Distilled water	100ml
Defidrinated goat blood	5ml - 10ml

A.8.2 Preparation methods

Heat to dissolve the above-mentioned components except for the fresh defidrinated goat blood, autoclave at 121℃ for 15 min, cool to 50℃, add defidrinated goat blood into the solution with aseptic techniques, shake well and then pour it into plates.

A.9 Sugar fermentation tube

A.9.1 Components

Beef extract	5.0g
Peptone	10.0g
Sodium chloride	3.0g
Dibasic sodium phosphate ($Na_2HPO_4 \cdot 12H_2O$)	2.0g

0.2% bromothymol blue solution	12.0ml
Distilled water	1000ml

A.9.2 Preparation methods

A.9.2.1 After the glucose fermentation tube is prepared with the above-mentioned components, add 0.5% glucose into it, dispense the solution to a small test tube with an inverted tube, adjust pH to 7.4, autoclave at 121℃ for 15 min, and then store it for later use.

A.9.2.2 Other kinds of sugar fermentation tubes After the tubes are prepared with the above-mentioned components, dispense the solution to tubes, 100ml per tube, autoclave at 121℃ for 15 min. Prepare 10% solutions of various carbohydrates and autoclave. Add 5ml sugar solution to 100ml culture medium, and dispense the solution to test tube with aseptic techniques.

A.9.3 Test methods

Inoculate sufficient amount of the pure culture to a sugar fermentation tube, incubate it at 36℃±1℃ for 24h - 48h, and observe the results; if the color is blue, the result is negative; if it is yellow, the result is positive.

A.10 Catalase test

A.10.1 Reagents

3% hydrogen peroxide solution: it should be prepared immediately before use.

A.10.2 Test methods

Pick a single colony with thin glass rod or single-use inoculation pin to a clean test tube, add 2ml 3% hydrogen peroxide solution drop by drop, and then observe the results.

A.10.3 Results

If there are bubbles generated within 30s, the result is positive; otherwise it is negative.